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**Antimicrobial evaluation of the polyisoprenylated benzophenones
nemorosone and guttiferone A**

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Polyisoprenylated benzophenones have been isolated from plants, particularly in the Clusiaceae family, and their biological properties have recently received considerable attention from a pharmacological point of view. The aim of the study was to investigate the polyisoprenylated benzophenones nemorosone and guttiferone A for their antimicrobial effect against a panel of bacteria, fungi and protozoan parasites. They showed a moderate activity against the Gram-positive bacterium *Staphylococcus aureus*, while no activity was demonstrated against *Escherichia coli* and the fungi *Trichophyton rubrum* and *Candida albicans*. An interesting activity was found for *Plasmodium falciparum* with IC₅₀ values lower than 1 µM, while cytotoxicity on MRC-5 cells revealed CC₅₀ values of 15.5 and 12.0 µM respectively for nemorosone and guttiferone A.

Keywords: nemorosone; guttiferone A; antibacterial; antiprotozoal.

INTRODUCTION

Infectious diseases are still a major threat to public health despite the tremendous progress in human medicine and their control remains a huge challenge since vaccines are only available against a limited number of pathogens. Most of the current anti-infectives suffer from considerable limitations in terms of antimicrobial spectrum and side-effects, and their widespread overuse has led to drug resistance (Fauci, 2001). Research on new anti-infectives must therefore be continued and all possible strategies should be explored.

In the area of infectious diseases, 75% of new drugs originated from natural sources between 1981 and 2002 (Newman *et al.*, 2003). As less than 10% of the world's biodiversity has yet been tested for biological activity, many more natural lead compounds are awaiting discovery (Bedoya *et al.*, 2009). The plant kingdom has been used as medicines for thousands of years and is undoubtedly a valid source of drug leads. They offer structural diversity that is not rivaled by the creativity or synthetic ingenuity of medicinal chemists. Well-known examples are taxol (paclitaxel) and camptothecin derivatives (topotecan and irinotecan) as antitumor agents and artemisinin derivatives (artemether and artesunate) as antimalarial agents (Koehn and Carter, 2005).

Polyisoprenylated benzophenones have been isolated from plants, particularly in the Clusiaceae family, and their biological properties have received considerable attention from a pharmacological point of view in recent years. These compounds are thought to be of mixed shikimate and acetate biosynthetic origin in which the acetate-derived benzene ring is modified by intervention of prenyl groups. Structural modifications of this ring results in the formation of complex molecules as bicyclo-[3.3.1]-nonane derivatives. Most of them show keto-enol equilibrium in solution and this hydroxyl group seems to play an important role in the biological effects of many

benzoylphloroglucinol derivatives including garcinol, xanthochymol and nemorosone (Cuesta-Rubio *et al.*, 2005). For several polyisoprenylated benzophenones, cytotoxic, antimicrobial, antioxidant and anti-inflammatory activities have been reported (Acuña *et al.*, 2009).

The aim of this study was to investigate the polyisoprenylated benzophenones nemorosone (Fig. 1A) and guttiferone A (Fig. 1B) for their antimicrobial effect against a panel of bacteria, fungi and protozoan parasites.

MATERIALS AND METHODS

Extraction of products. Flowers of *Clusia rosea* were collected in Havana (Cuba) in February 2010 and a voucher specimen (No.705) was deposited in the Herbarium of the Instituto de Investigaciones Fundamentales de la Agricultura, Havana, Cuba. Floral resins were extracted with 90% EtOH. Following filtration, the extract was dried under reduced pressure to yield a yellowish gum. The residue was subjected to RP-HPLC (90% MeOH) and nemorosone was crystallized using a mixture of EtOH–H₂O (70%-30%; v-v). HPLC separations were performed on a Waters 590 series pumping system equipped with a Waters R401 refractive index detector and a 300 x 7.8 mm i.d. 10 µm µ-Bondapak C-18 column (Waters). The structure of nemorosone was confirmed by ¹H NMR, ¹³C NMR and ESI-MS spectra, and its purity was verified by HPLC-DAD (>99%). All spectroscopic data were consistent with those previously reported (Piccinelli *et al.*, 2009).

Guttiferone A was obtained from *Garcinia aristata* fresh fruits by the same procedure employed to obtain aristofenone (Cuesta-Rubio *et al.*, 2001): fresh fruits were extracted with *n*-hexane; the yellow solid obtained after concentration under reduced pressure was

purified by HPLC; successive crystallizations from hexane gave crystalline guttiferone A. 1D- and 2-NMR experiments were in agreement with NMR data previously reported (Gustafson *et al.*, 1992). The purity of the compounds was analyzed by HPLC-DAD and HPLC-MS and was found to be 98 %.

Microorganisms and cells. The following microorganisms were used in this study: *Escherichia coli* ATCC8739, *Staphylococcus aureus* ATCC6538, *Trichophyton rubrum* B68183, *Candida albicans* B59630, chloroquine-resistant *P. falciparum* K1, suramin-sensitive *T. b. brucei* Squib-427, nifurtimox-sensitive *T. cruzi* Tulahuen CL2, *L. infantum* MHOM/MA(BE)/67 and *L. amazonensis* MHOM/77BR/LTB0016. Cytotoxicity was tested with human simian virus 40-immortalized lung fibroblasts (MRC-5 SV₂ cells; European Collection of Cell Cultures, United Kingdom).

Reference drugs. Erythromycin and chloramphenicol were purchased from Sigma-Aldrich (Bornem, Belgium), while miconazole and flucytosine were kindly supplied by Janssen Pharmaceuticals (Beerse, Belgium). Benznidazol, chloroquine, miltefosine and suramine were kindly provided by WHO-TDR. Amphotericin B was purchased from Imefa (Havana, Cuba).

Test plate production. The experiments were performed in 96-well plates (Greiner, Germany) at four-fold dilutions in a dose-titration range of 64 µg/mL to 0.25 µg/mL. Dilutions were carried out by a programmable precision robotic station (BIOMEK 2000, Beckman, USA). Each plate also contained medium-controls (blanks: 0% growth), infected untreated controls (negative control: 100 % growth) and reference controls (positive control). All tests were run in duplicate.

Biological screening tests. The integrated panel of microbial screens for the present study and the standard screening methodologies were adopted as have been described by [Cos et al., 2006](#).

Antibacterial activity: *S. aureus* and *E. coli* were cultured at 37 °C in MHB (Mueller Hinton broth) medium. Assays were performed by adding 5×10^3 CFU /well. After 17 hours incubation, bacterial viability was assessed fluorimetrically after adding resazurin ([Räz et al., 1997](#)) for 30 min at 37°C. Fluorescence was measured using a GENios Tecan fluorimeter (excitation 530 nm, emission 590 nm).

Antifungal activity: *T. rubrum* and *C. albicans* were cultured in RPMI medium at 27 °C and 37 °C respectively. Assays were performed by adding 5×10^3 CFU /well. After 7 days (for *T. rubrum*) or 24 h (for *C. albicans*) incubation, fungal viability was assessed fluorimetrically after adding resazurin for 24 h at 27 °C for *T. rubrum* and for 4 h at 37 °C for *C. albicans*.

Antiplasmodial activity: Parasites were cultured in human A⁺ erythrocytes at 37 °C under a low oxygen atmosphere (3% O₂, 4% CO₂, and 93% N₂) ([Trager and Jensen, 1976](#)) in RPMI-1640 culture medium, supplemented with 0.5 (g/v) % AlbumaxTM. Infected human red blood cell suspension (1% parasitemia, 2% hematocrit) were added to each well with test compounds and incubated for 72 h. After incubation, test plates were frozen at -20 °C. Parasite multiplication was measured by the Malstat method ([Makler et al., 1993](#)). One hundred microliters of MalstatTM reagent were transferred in a new plate and mixed with 20 µL of the hemolysed parasite suspension for 15 min at room temperature. After addition of 20 µL NBT (2 mg/mL)/PES (0.1 mg/mL) solution and 2 h incubation at room temperature in the dark, absorbance was read at 655 nm

(Biorad 3550-UV microplate reader). The percentage growth inhibition was calculated compared to the blanks.

Antitrypanosomal activity: trypomastigotes of *T. brucei* were cultured at 37 °C and 5% CO₂ in Hirumi-9 medium (Hirumi and Hirumi, 1989), supplemented with 10% inactivated fetal calf serum (FCSi). Assays were performed by adding 1.5 x 10⁴ trypomastigotes/well. After 72 h incubation, parasite growth was assessed fluorimetrically by adding resazurin for 24 h at 37 °C. Amastigotes of *T. cruzi* were maintained on MRC-5 cells in minimal essential medium (MEM) supplemented with 20 mM L-glutamine, 16.5 mM sodium hydrogen carbonate and FCSi (5%) at 37 °C and 5% CO₂. To determine *in vitro* anti-trypanosomal activity, 4 x 10³ MRC-5 cells and 4 x 10⁴ parasites were added to each well of test plate with compound. After incubation at 37 °C for 7 days, parasite growth was assessed by adding the β-galactosidase substrate chlorophenol red β-D-galactopyranoside (Buckner *et al.*, 1996) for 4 h at 37 °C. The colour reaction was read at 540 nm and absorbance values were expressed as a percentage of the blank controls.

Antileishmanial activity: *L. infantum* amastigotes were collected from an infected donor hamster and used to infect primary peritoneal mouse macrophages (PMM). To determine *in vitro* antileishmanial activity, 3 x 10⁴ macrophages were seeded in each well of a 96-well plate. After 48 h incubation at 37 °C and 5% CO₂, the cells were washed and infected with *L. infantum* amastigotes at a multiplicity of 15 parasites per cell. Two hours after infection, pre-diluted compounds were added and the plates were further incubated for 120 h at 37 °C and 5% CO₂. Then, the cells were fixed with methanol and stained with 10 % Giemsa for microscopic reading. The total parasite burden (= average number of amastigotes per cell) in treated wells was compared with control wells. For *L. amazonensis*, PMM were harvested and plated at 10⁶/mL in a 24-

well Lab-Tek (Costar[®], USA) and incubated at 37 °C and 5% CO₂ for 2 h. Non-adherent cells were removed and stationary-phase promastigotes were added at a 4:1 parasite/macrophage ratio for 4 h. The cells were washed to remove free parasites, pre-diluted compounds were added and the plates were further incubated for 48 h (Caio *et al.*, 1999). The total parasite burden was determined as described above.

Cytotoxicity assay: MRC-5 cells were cultivated in MEM, supplemented with L-glutamine (20 mM), 16.5 mM sodium hydrogen carbonate and 5 % FCSi at 37 °C and 5% CO₂. For the assay, 10⁴ MRC-5 cells/well were seeded onto the test plates containing the pre-diluted compounds and incubated at 37 °C and 5% CO₂ for 72 h. Cell viability was assessed fluorimetrically 72 h after addition of resazurin. For the highest antimicrobial activities, selectivity indices (SI) were calculated by the ratio of the CC₅₀ for MRC-5 cells and the IC₅₀ for microorganism.

Statistical Analysis. In each experiment, the 50 % of microbial growth (IC₅₀) and human cell growth (CC₅₀) inhibition value was determined from the lineal concentration-response curves and the results were expressed as the mean ± standard deviation of two independent experiments.

RESULTS

Nemorosone and guttiferone A were profiled *in vitro* for their antimicrobial activities. The benzophenones showed a moderate activity against the Gram-positive bacterium *S. aureus*, while no activity was demonstrated against *E. coli* and the fungi *T. rubrum* and *C. albicans* (Table 1). More interesting results were obtained against protozoan parasites (Table 2). The highest activity was found for *P. falciparum* with IC₅₀ values lower than 1 µM. The cytotoxicity on MRC-5 cells revealed CC₅₀ values of 15.5 ± 2.9

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2
3 μM and $12.0 \pm 1.0 \mu\text{M}$ for respectively nemorosone and guttiferone A. In general, a low
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5 selectivity was found, except for *P. falciparum* with an SI value of 39 for nemorosone
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7 and 24 for guttiferone A.
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10 11 12 13 DISCUSSION

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15 Plants are an important source in the search of new and selective drugs for the
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17 treatment of infectious diseases. The antimicrobial activity of plant-derived agents has
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19 been attributed to compounds belonging to diverse chemical groups, such as alkaloids,
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21 saponins and terpenes (Alviano and Alviano, 2009). Benzophenones represent another
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23 interesting class of bioactive compounds with anti-inflammatory, antioxidant and
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25 antitumoral effects (Acuña *et al.*, 2009). In addition, several benzophenones have been
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27 shown to possess antibacterial activities, like peroxysampsonone A (Xiao *et al.*, 2010),
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29 garcinol (Rukachaisirikul *et al.*, 2005) and microsphaerin (Yoganathan *et al.*, 2008)
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31 against *S. aureus*. Antiplasmodial effects have also been demonstrated, for example for
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33 coccinone, isogarcinol and symphonone (Marti *et al.*, 2010).
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39 In the current study, the natural benzophenones nemorosone and guttiferone A were
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41 evaluated against a wide panel of microorganisms, including a Gram-negative (*E. coli*)
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43 and a Gram-positive (*S. aureus*) bacterium, a dermatophyte (*T. rubrum*), a yeast (*C.*
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45 *albicans*) and protozoa that cause relevant parasitic diseases, namely malaria (*P.*
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47 *falciparum*), Chagas disease (*T. cruzi*), sleeping disease (*T. brucei*), and visceral (*L.*
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49 *infantum*) and cutaneous (*L. amazonensis*) leishmaniasis.
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53 Nemorosone has been isolated from plants, like *Tovomitopsis saldanhae* (Bitttrich *et*
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55 *al.*, 2003) and *Clusia grandiflora* (Lokvam *et al.*, 2000), as well as from propolis
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57 (Cuesta-Rubio *et al.*, 2002, Tomás-Barberán 1993). Antitumoral, antioxidant (Cuesta-
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59 Rubio *et al.*, 2002) and antibacterial activity against *Salmonella typhi*, *Proteus*
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3 *mirabilis*, *Shigella sonnei* and *Streptococcus faecalis* (Rojas *et al.*, 2001) have been
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5 documented. In addition, activity against honeybee pathogens *Paenibacillus larvae* and
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7 *Paenibacillus alvei* was also reported (Lokvam *et al.*, 2000).
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10 Benzoylphloroglucinol derivatives isolated from different plants have been named
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12 guttiferones A-F, H and O. Guttiferone A was first isolated from *Symphonia globulifera*
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14 as an anti-HIV constituent (Gustafson *et al.*, 1992). Other studies have described their
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16 isolation from *Garcinia brasiliensis* (Pereira *et al.*, 2010) and *Allanblackia monticola*
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18 (Lenta *et al.*, 2007) and antiproliferative effect on *T. cruzi* (Abe *et al.*, 2004), *L.*
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20 *amazonensis* (Pereira *et al.*, 2010) and *P. falciparum* (Ngouela *et al.*, 2006). In parallel,
21
22 a potent free radical scavenging activity compared to the well-known antioxidant caffeic
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24 acid was observed (Ngouela *et al.*, 2006).
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29 Except for *P. falciparum*, an unspecific activity was observed as demonstrated by the
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31 low SI. Both compounds were inactive against *E. coli* and *C. albicans*, which
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33 correspond with previous reports (Naldoni *et al.*, 2009). The highest activity for the two
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35 benzophenones was demonstrated against *P. falciparum*, with IC₅₀ values below 1 µM
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37 and with SI values of 39 for nemorosone and 24 for guttiferone A. The mechanism of
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39 action of benzophenones has not been studied in detail. Nevertheless, guttiferone A
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41 caused a potent *in vitro* inhibition of cysteine protease cathepsins B and papain and
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43 serine peptidases cathepsins G and trypsin (Martins *et al.*, 2009), which are present in
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45 *Plasmodium* parasites and play an important role in their survival and development in
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47 host cells (Rosenthal, 2004). Further experiments in animal models of malaria should be
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49 performed with the aim to validate these compounds as potential antimalarial drug
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51 leads. Benzophenones could be used as a pharmacophore group to design new
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53 prenylated benzophenone derivatives. This was shown in previous structure-activity
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55 studies where the biological activity increased proportionally with the number of prenyl
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57
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groups and consequently with their lipophilicity (Pereira *et al.*, 2010; Urzúa *et al.*, 2008). Previous studies have demonstrated that the more lipophilic benzophenones showed the highest activity against *Leishmania* parasites. This could be correlated with an increased capacity to cross the macrophage and to reach the amastigote form (Pereira *et al.*, 2010). Other benzophenones displayed toxicity and non-specific action such as symphonone H (Marti *et al.*, 2010), 2,5-diaminobenzophenone (Altenkämper *et al.*, 2009), guttiferone O and guttiferone E (Lannang *et al.*, 2010).

Although some studies are available on the antimicrobial activity of guttiferone A, to our knowledge, this is the first study on nemorosone against a wide panel of microbial pathogens. A selective activity was demonstrated against *P. falciparum* for nemorosone and guttiferone A, requiring for chemical derivatisation and further exploration in animal models.

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LEGEND OF FIGURE 1

Figure 1. Chemical structures of polyisoprenylated benzophenones used in this study.

A: Nemorosone; B: Guttiferone A

Table 1. Activity of nemorosone and guttiferone A against bacteria and fungi.

Products	IC ₅₀ ^a ± SD ^b (μM)			
	<i>S. aureus</i>	<i>E. coli</i>	<i>T. rubrum</i>	<i>C. albicans</i>
Nemorosone	16.1 ± 0.1	> 64.00	> 64.00	> 64.00
Guttiferone A	7.5 ± 0.8	> 64.00	19.6 ± 17.5	> 64.00
Erythromycin	6.6 ± 4.3	-	-	-
Chloramphenicol	-	6.9 ± 3.4	-	-
Miconazole	-	-	0.3 ± 0.2	-
Flucytosine	-	-	-	0.7 ± 0.1

^a: IC₅₀: Concentration of compound that caused 50 % of inhibition growth

^b: SD: Standard deviation

Table 2. Activity of nemorosone and guttiferone A against protozoa.

Products	IC ₅₀ ^a ± SD ^b (µM)				
	<i>P. falciparum</i>	<i>T. cruzi</i>	<i>T. brucei</i>	<i>L. infantum</i>	<i>L. amazonensis</i>
Nemorosone	0.4 ± 0.2	12.5 ± 1.7	17.5 ± 1.9	32.9 ± 5.4	11.2 ± 0.6
Guttiferone A	0.5 ± 0.2	11.8 ± 1.1	13.5 ± 0.1	13.5 ± 0	15.6 ± 0.1
Chloroquine	0.3 ± 0.1	-	-		-
Benznidazol	-	2.2 ± 0.5	-		-
Suramine	-	-	0.05 ± 0.05		-
Miltefosine	-	-		7.7 ± 4.5	-
Amphotericin B	-	-			0.03 ± 0.006

^a: IC₅₀: Concentration of compounds that caused 50 % of inhibition growth

^b: SD: Standard deviation

254x142mm (96 x 96 DPI)